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In vitro regeneration and assessment of genetic fidelity of acclimated plantlets by using ISSR markers in PPR-1 (*Morus* sp.): An economically important plant

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ABSTRACT

Mulberry is a tree species; hence, it is difficult to carry out the indirect generation. In view of this limitation, in present study an indirect regeneration protocol was developed for *in vitro* clonal propagation of sericulturally important superior temperate mulberry variety cv. Pampore-1 (PPR-1) by using leaf, petiole and nodal based callus. Initially friable callus (mean fresh weight) was induced in maximum amounts (288.2 ± 21.09; 248.2 ± 16.52 & 138.4 ± 04.25) from leaf, petiole and nodal explants on Murashige and Skoog (MS) media supplemented with 5.0 µM/L, 7.5 µM/L of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 12.5 µM/L of Naphthalene acetic acid (NAA), respectively, after 3 weeks of culture. Best response of shoot regeneration from the induced callus with maximum frequency (14.8 ± 0.82) was observed in leaf callus on MS media supplemented with 2.5 µM/L of Thidiazuron (TDZ) + 2.5 µM/L of 6-Benzyl amino purine (BAP). The regenerated shoots were rooted with maximum rooting frequency (96 ± 02.2) on MS media with 7.5 µM/L concentration of Indole-3-Butyric acid (IBA). The raised plantlets were then hardened using 1:1:2 ratio of farmyard manure, sand and garden soil, then gradually they were transferred and acclimatized to field conditions. The survival rate of *in vitro* raised PPR-1 plantlets in field conditions is about 82%. Genetic homogeneity between the micropropagated plants and mother plant was confirmed by carrying out the inter simple sequence repeats (ISSR) primer based polymerase chain reaction (PCR) analysis. Among the ten ISSR primers used, two primers, *i.e.*, M5 and M8 has given good amplification with clear, distinct and scorable DNA bands which were monomorphic across the micropropagated plantlets and the mother plant. Hence, the *in vitro* regenerated PPR-1 mulberry plantlets were confirmed as clonally uniform and genetically stable.

1. Introduction

Mulberry (*Morus* spp.) is described as exceptional leaf foliage (Sanchez, 2000, 2002) due to its multiple uses and its availability all over the world. Besides its usage as feeding material for silk worm (Miyashita, 1986; Rahmathulla, 2012; Vineet et al., 2012; Gandhi et al., 2012) and several animals (Prasad et al., 2003; Anbarasu et al., 2004; Martinez et al., 2005; Bakshi and Wadhwa, 2007; Kandylis et al., 2009); it is also used as a protein source for pharmaceutical formulations (Butt et al., 2008), in treatment of diabetes (Ewelina et al., 2016), as a neuroprotective (Niidome et al., 2007), to reduce the triglycerides in body (Andallu et al., 2001), for neutralizing the effects of free radicals (Naowaratwattana et al., 2010); for the skin improvement (Lee et al., 2002; Fang et al., 2005).

Jammu & Kashmir (J&K) is one of the traditional sericulture states in India which comprises of sub-tropical (Jammu Division), temperate (Kashmir Division) and cold arid (Ladakh Division) climatic conditions (Gani et al., 2016) and traditionally this state is well known for the quality bivoltine type of mori silk production across the country (Maqbool et al., 2015). This state is also well-known for the existence of several mulberry species such as *Morus alba*, *M.indica*, *M.laevigata*, *M.serrata, M.nigra* and *M.rubra* (Bindroo et al., 2005; Aftab et al., 2016).

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BmNPV, *Bombyx mori* nucleopolyhedrovirus; BAP, 6-Benzyl aminopurine; cm, centimeter; IAA, Indole-3-Acetic Acid; IBA, Indole-3-Butyric Acid; ISSR, inter simple sequence repeats; Kn, Kinetin; NAA, Naphthalene Acetic Acid; PPR-1, Pampore-1; TDZ, Thidiazuron

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Central Sericulture Research & Training Institute (CSR&TI), Pampore (J &K) has recently developed an interspecific hybrid PPR-1 (Pampore-1), a superior temperate mulberry variety evolved through controlled hybridization by utilizing Goshoerami Goshoerami (*M.multicaulis*) and Chinese white (*M.alba* L.) mulberry varieties as parental lines.

PPR-1 variety is superior to the existing ruling varieties of temperate region in terms of rooting ability, frost tolerance, leaf yield, leaf quality, moisture content and moisture retention capacity (Afatb et al., 2014). Based on its improved characteristics, PPR-1 variety was released in 2016 for its commercial exploitation in sericulture sector of temperate regions across the North West India (Aftab and Sharma, 2016).

Among the different types of mori silk, bivoltine silk is compared as superior based on its reeling characters, shell ratio and other parameters. In spite of having favourable conditions for bivoltine cocoon production and having quality leaf of PPR-1, the silk production in temperate regions is lesser than the sub-tropical and tropical regions of India; it is mainly due to unavailability of mulberry leaf foliage during cold predominant winter season nearly for 5–6 months duration (from November to April) and due to the impact of BmNPV infection (Gani et al., 2017). Hence, the silk production in this region is limited mainly to spring season and to some extent to autumn season, where as in tropical region of India, the silkworm rearing and mori silk production is carried out for 5–8 crops per year due to continuous availability of mulberry leaf foliage (Vijayan et al., 2000; Rahmathulla, 2012).

Due to prevailing cold conditions and genetic makeup of the temperate mulberry accessions, the propagation of mulberry in temperate climate has remained a major drawback over the years (Aftab et al., 2012; Rohela et al., 2016b). The cold climatic conditions decrease the soil temperature and in turn effect the root formation. Besides this, long dormancy period of six months (October to March) hinders the growth of saplings. Due to these reasons, conventionally it takes 3–5 years for a mulberry sapling to be ready for transplantation in temperate region (Shamim and Asharaf, 2001) in comparison of 6 months to 1 year at tropical regions (Krishnaswamy, 1986; Datta, 2000). In temperate regions, this long duration of preparing mulberry saplings can be drastically reduced if the winter dormancy period can be utilized for *in vitro* propagation of mulberry.

In vitro micropropagation in combination with green house can rapidly multiply and provide the mulberry saplings to spread the sericulture sector in this region. There are several reports about the *in vitro* micropropagation of tropical mulberry varieties and few reports in temperate mulberry varieties through the shoot tip, nodal and axillary bud explants (Patel et al., 1983; Jain et al., 1990; Chattopadhyay et al., 1990; Sharma and Thrope, 1990; Yadav et al., 1990; Rao and Bapat, 1993; Tewary et al., 1996; Tewary and Subba, 2000; Zaman et al., 1997; Pattnaik and Chand, 1997; Chitra and Padmaja, 1999; Zaki et al., 2011; Alatar et al., 2003; Sajeevan et al., 2011; Anis et al., 2003; Kavyashree, 2007; Attia-o-Attia et al., 2014).

Mulberry is a tree species, therefore it is rather difficult to carryout the indirect generation, because the callus of most of the tree species produce phenols, alkaloids and secondary metabolites in *in vitro* conditions which convert the callus to compact and non-friable type. It is very difficult to regenerate the shoots from compact callus. In spite of this difficulty, some researchers have reported about the indirect regeneration in tropical mulberry varieties (Oka and Ohyama, 1985; Yamanouchi et al., 1999; Vijayan et al., 2000; Bhau and Wakhlu, 2001; Prasada et al., 2010; Kathiravan et al., 2013), but till now to the best of our knowledge, there is no report on indirect regeneration in temperate mulberry.

In view of above limitations, the present study is undertaken with the objective of raising the superior temperate mulberry variety cv. PPR-1 through the indirect regeneration protocol by utilizing leaf, petiole and nodal segments as explants. Indirect regeneration in a temperate mulberry is reported for the first time through this research paper. The developed protocol will reduce the duration of sapling raising under temperate climatic conditions from the existing 3–5 years to 1 year and more importantly, the non-productive winter months can be utilized for raising the saplings under laboratory conditions which can be transferred to the land during the early onset of spring (March–April). The protocol will also be utilized in future research works related to improvement of mulberry through genome editing and genetic transformation experiments during which callus based regeneration of shoots has to be carried out.

2. Materials and methods

2.1. Plant material and explant source

Three years old PPR-1 mulberry plants available in the CSR&TI, Pampore Institute were used as parent plant material in these *in vitro* propagation studies. Leaf and nodal segments were collected during the third week of April from these plants and used as explants for the study.

2.2. MS media preparation

Full strength MS medium (Murashige and Skoog, 1962) was prepared with 30 g/l sucrose. Before dispensing the prepared medium to culture vessels, required concentrations and combinations of plant growth regulators were added to it from the hormonal stock solutions. The pH of the medium was adjusted to 5.6 using NaOH solution before autoclaving. The medium was solidified with 0.8% agar.

2.3. Explant surface sterilization

Leaf and nodal explants of PPR-1 were collected from the mulberry field and initially washed under running tap water. Then different protocols were followed for the sterilization of leaf and nodal explants, as leaf explants are more delicate and sensitive in nature to sterilants. Leaf explants were treated with Tween-20 solution for 1 min followed by 45% ethanol for 2 min and in 0.1% mercuric chloride ($HgCl_2$) solution for 2 min. Nodal explants were treated with Tween-20 solution for 2 min followed by 0.5% sodium hypochlorite solution for 2 min, with 60% ethanol for 2 min and with 0.1% mercuric chloride solution for 3 min. After every treatment explants were rinsed in sterile distilled water to remove the adhering chemical sterilizing agents.

2.4. Inoculation and incubation

Surface sterilized leaf and nodal explants of PPR-1 were inoculated onto MS medium present under aseptic conditions of laminar air flow cabinet with the help of sterile forceps. Culture vessels with inoculated explants were kept in culture room under controlled conditions of 16/8 h of photoperiod with 3000 lx intensity light at 26 ± 2 °C.

2.5. Callus induction

For callus induction leaf, petiole and nodal explants of PPR-1 were inoculated on to MS medium with 2,4-D or NAA (2.5–15.0 μM/L) (Tables 1 and 2).

2.6. Shoot regeneration

For shoot regeneration from the friable callus, cytokinins (Kn, BAP and TDZ) supplemented MS media was prepared in individual (Table 3) and combinations. When cytokinins were used individually, good response of shooting was observed at 2.5 μM/L concentrations of TDZ. Hence when combination of cytokinins was made 2.5 μM/L of TDZ was kept constant with increasing concentrations of Kn or BAP.

2.7. Rooting of the regenerated shoots

The regenerated shoots were separated from the culture tubes and